High molecular weight proteins in the nematode *C. elegans* bind [^{3}H]ryanodine and form a large conductance channel

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ABSTRACT Single-channel properties of a polypeptide fraction from the nematode *Caenorhabditis elegans* highly enriched in binding sites were studied in planar bilayers. [^{3}H]Ryanodine binding sites were purified by sucrose gradient centrifugation of *C. elegans* microsomes solubilized in CHAPS detergent. The highest [^{3}H]ryanodine binding activity sedimented at ~18% sucrose (wt/vol), and was composed of a major polypeptide with a M_r of 360,000 and a minor polypeptide with a M_r of 170,000. The ryanodine-binding polypeptide(s) formed a Ca^{2+}-permeable channel with a permeability ratio P(divalent)/P(monovalent) = 4 and two conductance states of 215 pS and 78 pS in 0.25 M KCl. Ryanodine locked the channel in the 78 pS state and inhibited transitions between the 215 pS and 78 pS states. These data demonstrated the presence of a ryanodine receptor in *C. elegans* with functional properties comparable to those described in mammalian muscle.

INTRODUCTION

The ryanodine receptor forms a channel that releases Ca^{2+} from the sarcoplasmic reticulum (SR) of mammalian striated muscle in response to electrical or chemical stimulation of muscle fibers (1). Both the ligand-dependent gating of this channel by Ca^{2+} and the voltage-dependent gating by voltage sensors located in the transverse tubular membrane are thought to play a crucial role in excitation–contraction (EC) coupling (2). The morphological unit that binds ryanodine (3, 4) and functions as a Ca^{2+} channel is a homotetramer with a sedimentation coefficient of approximately 30 S (5). A three-dimensional reconstruction of the 30 S receptor suggests a structure in which the four monomers form one central channel on the lumenal side of the SR and branch out to form four radial channels on the cytoplasmic side of the SR (6). Conductance and gating characteristics of ryanodine receptors have been investigated in a variety of mammalian tissues, including skeletal muscle, myocardium, and brain (7–10). A cardiac isoform, composed of 4,969 amino acids (564.7 kD) is expressed in heart and brain, whereas a skeletal isoform, composed of 5,037 amino acids (565.2 kD) is expressed in both fast- and slow-twitch skeletal muscle (11, 12).

Whether these isoforms, or perhaps others, exist in invertebrate muscle is presently unknown. To address this question, we investigated the presence of ryanodine receptors in a genetically accessible invertebrate, the nematode *Caenorhabditis elegans*. Little is currently known about EC coupling in *C. elegans*. Locomotion of the animal is controlled by excitatory and inhibitory motor neurons, which synapse with appropriate body-wall muscle cells along the length of the animal (13). Coordinated contraction and relaxation of dorsal/ventral groups of cells generates a wave-like motion that propels the animal forward or backward. *C. elegans* muscle is obliquely striated (14), like that of other invertebrates (15), and contains an array of myofilament proteins that is typical of invertebrates (16, 17). *C. elegans* exhibits both thin and thick-filament-linked Ca^{2+} regulatory systems (18). Thin filaments attach to dense bodies, which are structurally analogous to the Z-discs of vertebrate skeletal muscle. The *C. elegans* SR consists of a network of membranous vesicles and sheet-like sacs that surround dense bodies and extend towards and along the cytoplasmic face of the plasma membrane (16). *C. elegans* muscle cells are much smaller and thinner than those of vertebrates, and a distinct system of plasma membrane-derived transverse tubules is not present. Rather, the SR is closely apposed to the plasma membrane itself. *C. elegans* ryanodine receptors, if present, would likely be located within the membranous SR where, following excitation of muscle cells, they could mediate the release of Ca^{2+}. We show that [^{3}H]-ryanodine binding sites can be purified from a wild-type strain of *C. elegans* and upon incorporation into planar bilayers, they form ryanodine-sensitive Ca^{2+}-permeable channels similar to those of mammalian muscle and brain. Hence, the presence of a functional ryanodine receptor was established in *C. elegans*. Part of this work appeared in abstract form (19).

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MATERIALS AND METHODS

Nematode strains and culture. The conditions for growth and maintenance of *C. elegans* have been described (20). The wild-type strain N2 was used for all experiments. Gram quantities of *C. elegans* were grown in 1-liter batches at 20°C. *Escherichia coli* bacteria from 2 liters of stationary culture were pelleted, combined with 1 liter of S-media (20), and inoculated with $\approx 10^4$ nematodes. Cultures were grown with vigorous aeration until the bacteria were exhausted. Animals were allowed to settle overnight at 4°C, collected in 50-ml tubes, washed in M9 buffer (20), and floated by centrifugation on ice cold 35% sucrose prior to collection. The floated animals were washed again in M9 buffer and

quickly frozen in liquid nitrogen. Wet weight yield ranged from 4 to 7 g/liter.

Purification of ryanodine receptor. Pellets of frozen *C. elegans* (5–20 g wet weight) were resuspended in 80 ml of a low-salt homogenization buffer consisting of 5 mM Tris-Maleate (pH 7.2) plus the following protease inhibitors: Pepstatin A (1 μM); Iodoacetamide (1 mM); PMSF (0.1 mM); Leupeptin (1 μM); and Benzamidine (1 mM). The suspension was sonicated on ice for 10 min using a bath-type sonicator (80 W; LSI Co., Hicksville, NY), homogenized with a motor-driven glass–teflon homogenizer at low speed, and centrifuged at 2,400 *g* for 30 min. The supernatant was collected and centrifuged at 80,000 *g* for 40 min. Pelleted membranes were resuspended in 0.6 M KCl, 5 mM Tris-Mes (pH 6.8) plus the protease inhibitors described above. This material was incubated on ice for 30 min and loaded on top of a discontinuous sucrose gradient composed of 20%, 30%, and 40% sucrose in 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-Mes (pH 6.8). Gradients were centrifuged for 5 h at 90,000 *g*, and fractions were collected from between sucrose layers and diluted with ice-cold glass-distilled water to about 10% sucrose. Fractions were pelleted by centrifugation at 90,000 *g* for 40 min and assayed for [^{3}H]ryanodine binding activity. Pelleted membranes from the 30 to 40% sucrose region were resuspended in 1 ml of 0.5% CHAPS, 1 M NaCl, 40 mM Tris-Maleate (pH 7.2), and incubated on ice for 30 min. Solubilized material was layered on top of a linear sucrose gradient consisting of 5 to 20% sucrose in 0.3 M NaCl, 0.1 M $CaCl_2$, 2 mM dithiothreitol, 0.15% CHAPS, 40 mM Tris-Maleate (pH 7.2), and centrifuged at 60,000 *g* in an SW-28 rotor (Beckman Instruments, Inc., Fullerton, CA) for 16 h. Gradients were fractionated into 1.5-ml aliquots. Protein concentration was determined by the Lowry method, using bovine serum albumin as standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels consisted of a 4–12% polyacrylamide gradient in Laemmli buffers. Gels were stained with a silver-staining kit from Bio-Rad Laboratories (Richmond, CA). Molecular weights of the standards were: myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine albumin, 68,000; ovalbumin, 43,000.

[^{3}H]ryanodine binding assay. Sucrose gradient fractions were incubated with 7 nM [^{3}H]ryanodine for 1.5 h at 36°C in a solution containing either 0.3 M NaCl, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, 0.15% CHAPS, 40 mM Tris-Maleate (pH 7.2) in the case of solubilized receptors; or 0.2 M KCl, 1 mM EGTA, 1 mM $CaCl_2$ (10 μM free Ca^{2+}), 20 mM Na-Pipes (pH 7.2) in the case of microsomes. [^{3}H]Ryanodine (60 mCi/nmol) was diluted directly into the incubation medium to achieve a final concentration in the range of 1 to 50 nM. Each sample was measured in triplicate. Samples were filtered onto glass fiber filters (GF/B or GF/C; Whatman Inc., Clifton, NJ) presoaked in 5% polyethylenimine for 15 min, and washed twice with 5 ml of glass-distilled water. The nonspecific [^{3}H]ryanodine binding was defined as the binding activity in the presence of 2 μM unlabeled ryanodine.

Single-channel recordings. Planar lipid bilayers were formed on a 0.42-mm diameter hole in a Delrin cup. The bilayers were cast from a solution of brain phosphatidylethanolamine and phosphatidylserine mixture (20 mg/ml) at a 1:1 ratio dissolved in decane. Solubilized receptors (5–10 μg) were added to the *cis* chamber solution containing 1 to 3 ml of 250 mM KCl, 10 mM Hepes-Tris (pH 7.2). The trans solution (3 ml) was the same. Cationic selectivity was determined by changes in reversal potential before and after *trans* addition of 5 mM $CaCl_2$ to channels recorded in *cis* 250 mM Cs_2SO4, 10 mM Hepes-Tris (pH 7.2) and *trans* 50 mM Cs_2SO_4, 10 mM Hepes-Tris pH (7.2). All solutions contained an estimated free Ca^{2+} of 1 to 3 μM present as a contaminant. Current was filtered through a low-pass Bessel filter (Frequency Devices Inc., Haverhill, MA) at 0.3 to 1 kHz. Data were analyzed by pClamp 5.5 software (Axon Instrument, Foster City, CA).

Chemicals and abbreviations. Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Ryanodine was obtained from Calbiochem Corp. (La Jolla, CA). [^{3}H]Ryanodine (60 mCi/nmol) was purchased from Du Pont-New England Nuclear Co. (Boston, MA). CsCl and CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) were purchased from Sigma Chemical Co. (St. Louis, MO). PMSF (Phenylmethylsulfonyl fluoride); Mes (2-[*N*-Morpholino] ethanesulfonic acid); Pipes (Piperazine-*N,N'*-bis[2-ethanesulfonic acid]).

RESULTS AND DISCUSSION

Ryanodine induces partial paralysis and hypercontraction of wild-type *C. elegans,* implying that ryanodine receptor channels may be present in this organism. In our hands, maximal effects were observed at $\geq$100 μM ryanodine added to the growth medium. This alkaloid concentration was much higher than that needed to induce contraction of isolated mammalian muscle, which is typically 1 to 10 μM (21). However, it was considered reasonable for a paralysis in vivo because the alkaloid must diffuse across several tissue layers before reaching muscle cells within *C. elegans.* Following disruption of nematodes by osmotic shock and sonication, microsomes were separated on a discontinuous sucrose gradient and assayed for [^{3}H]ryanodine binding activity. Fig. 1 *A* shows the specific binding of [^{3}H]ryanodine to microsomes sedimenting between 30 and 40% sucrose (vol/wt), the fraction containing the highest binding activity. Binding saturated at approximately 20 nM [^{3}H]-ryanodine, yielding a single-site density (B_{max}) of approximately 40 fmol/mg protein and a dissociation constant (K_d) of 26 nM. Purification of binding sites was achieved by solubilization in 0.3 M NaCl, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, 0.15% CHAPS, 40 mM Tris-Maleate (pH 7.2) followed by centrifugation on a linear gradient of 5% to 20% sucrose. As shown in Fig. 1 *B,* the bulk of the protein solubilized from the 30–40% sucrose fraction remained on top of the gradient (<13% sucrose) while most of the [^{3}H]ryanodine binding activity sedimented as a sharp peak between 18% and 19% sucrose (wt/vol). The sedimentation profile of the [^{3}H]ryanodine binding sites of *C. elegans* was similar to that described for mammalian ryanodine receptors (5, 8, 9).

The [^{3}H]ryanodine binding characteristics of the solubilized receptor recovered from the sucrose gradient are shown in Fig. 1 *C.* Using conditions optimized for the mammalian receptor (22), we found that binding saturated with a B_{max} of approximately 110 fmol/mg protein and a K_d of approximately 26 nM. The K_d of the purified receptor was similar to that of *C. elegans* microsomes and similar also to that of ryanodine receptors of striated muscle and brain (5, 8, 9). The B_{max} of the purified receptor in three separate preparations was three to five-fold higher than that present in the original microsomal fraction and 10 to 12-fold higher than that of the crude homogenate (see Table 1). A similar purification procedure using rabbit skeletal muscle yields a typical enrichment, from whole muscle homogenate to the CHAPS-solubilized 18% sucrose fraction, of 200-fold (22). We attributed the lower enrichment in *C. elegans* to the fact

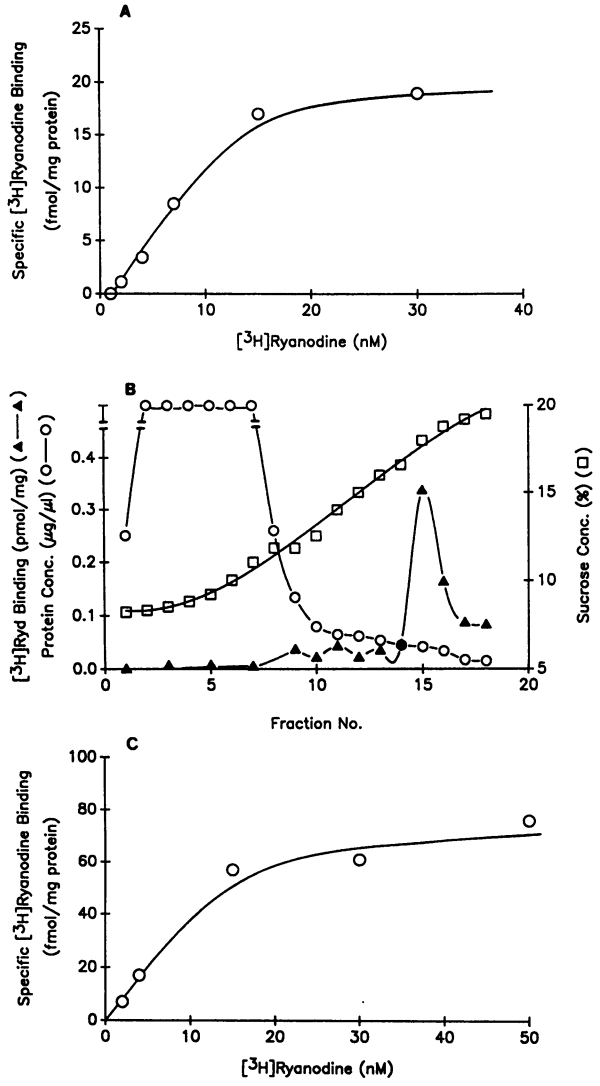

FIGURE 1 [^{3}H]Ryanodine binding to *C. elegans* microsomes and CHAPS-purified receptor. (*A*) Saturation of specific [^{3}H]ryanodine binding to microsomal membranes sedimenting between 30 and 40% collected by discontinuous sucrose gradient centrifugation. (*B*) Purification of CHAPS-solubilized [^{3}H]ryanodine binding activity by linear sucrose gradient centrifugation. Fractions were collected from the top of a 5–20% linear gradient. Ryanodine binding activity of each fraction (*filled triangles*) was measured in the presence of 7 nM [^{3}H]ryanodine. Binding activity was contained in a single peak in fractions between 18 and 19% sucrose. (*C*) Saturation of specific [^{3}H]ryanodine binding to the CHAPS-solubilized sucrose gradient-purified ryanodine receptor-enriched fraction.

that [^{3}H]ryanodine binding activity following solubilization in CHAPS was extremely labile and decreased continuously in time, either in the presence or absence of protease inhibitors. Hence, it is conceivable that loss of binding activity during the sucrose gradient fractionation could account for the low enrichment in binding sites. The polypeptide pattern of the sucrose gradient-purified receptor is shown in Fig. 2. By SDS-PAGE analysis, the fraction containing the highest ryanodine-binding activity was composed of one major band with a M_r of 360,000 (lane *5*). A minor band with a M_r of 170,000 may represent a proteolytic fragment of the larger polypeptide (5). Polypeptides with a M_r in the range of 170,000 to 360,000 were also observed in the original microsomal fraction used for solubilization (lane *3*), and in the pellet under the 40% sucrose layer (lane *4*). However, only a few high molecular weight polypeptides were observed in microsomes sedimenting in less than 30% sucrose. The molecular mass of the large polypeptide purified from *C. elegans* is similar to that established by gel electrophoresis for mammalian ryanodine receptors. The cardiac ryanodine receptor purified from rabbit migrates with a M_r slightly less than 340,000 in the same gel system used here. However, the molecular mass of this receptor determined by sequence analysis is 564,711 Da (11). The molecular mass of the nematode ryanodine receptor could thus be larger than that estimated by its electrophoretic mobility. The sedimentation profile of the CHAPS solubilized receptor, the high-affinity binding of [^{3}H]ryanodine to the purified receptor, and the molecular weight of the purified receptor protein(s), suggested the presence of a bona fide ryanodine receptor in *C. elegans*.

Fig. 3 *A* shows planar bilayer recordings of the [^{3}H]-ryanodine binding peptide(s) in *cis* and *trans* solutions containing 250 mM KCl, 10 mM Hepes-Tris, pH 7.2. Monovalent cations were chosen as the current carrier to improve the signal-to-noise ratio. In K^+-containing solutions we observed two single-channel conductances of 78 ± 7 pS and 215 ± 13 pS. In three separate preparations, the probability of the 78 pS state was 0.9 ± 0.02 while that of the 215 pS state was 0.34 ± 0.2. Both states were observed in the range of −80 to +80 mV and displayed little voltage dependence. Two observations suggested that the 78-pS and the 215-pS conductance states originated from the same channel. First, an ≈290-pS channel generated by the simultaneous opening or closing of these two states was commonly observed. Second, the two conductances behaved in a nonindependent manner since following addition of solubilized receptor to the *cis* chamber, the 78-pS state was always the first to be recorded. The selectivity of the nematode channel for

TABLE 1 **Purification of *C. elegans* ryanodine receptors**

	Total protein	Total binding	Spec. activity	Purification	Yield
	mg	*fmol*	*fmol/mg*	*fold*	%
Whole nematode homogenate	950	9500	10	1.0	100
30–40% sucrose microsomes	80	3200	40	4.0	30
CHAPS-solubilized 18% sucrose peak	0.4	440	110	11	5

Entries correspond to a fractionation of 5 g (wet weight) of a pelleted culture of *C. elegans* (see Materials and Methods).

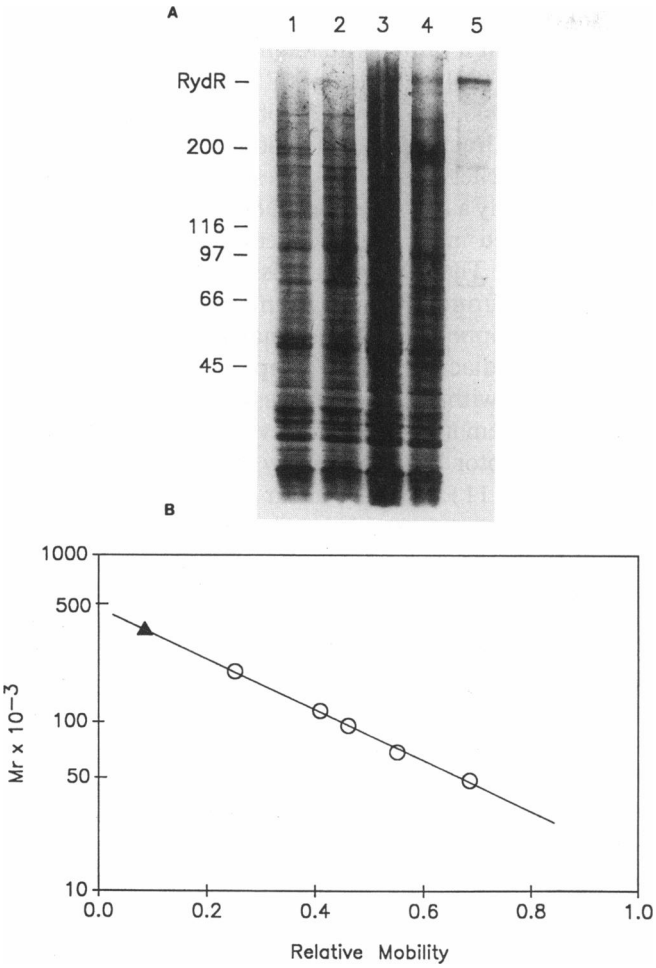

FIGURE 2 Polypeptide composition of ryanodine receptor from *C. elegans*. (*A*) Microsomal and purified receptor fractions were analyzed by 4 to 12% gradient SDS-PAGE. Lanes *1–4* correspond to *C. elegans* microsomes purified by sucrose gradient centrifugation. Collected interface between 10 and 20% sucrose (lane *1*); 20 and 30% sucrose (lane *2*); 30 and 40% sucrose (lane *3*); and pellet at the bottom of 40% sucrose (lane *4*). Lane *5* corresponds to the CHAPS-solubilized sucrose gradient-purified ryanodine receptor-enriched fraction. (*B*) The triangle corresponds to the relative mobility of the largest polypeptide of lane *5* (labeled RydR). This peptide migrated with an apparent molecular mass of ~360 kD. Open circles correspond to molecular weight markers indicated in (*A*) and described in Materials and Methods.

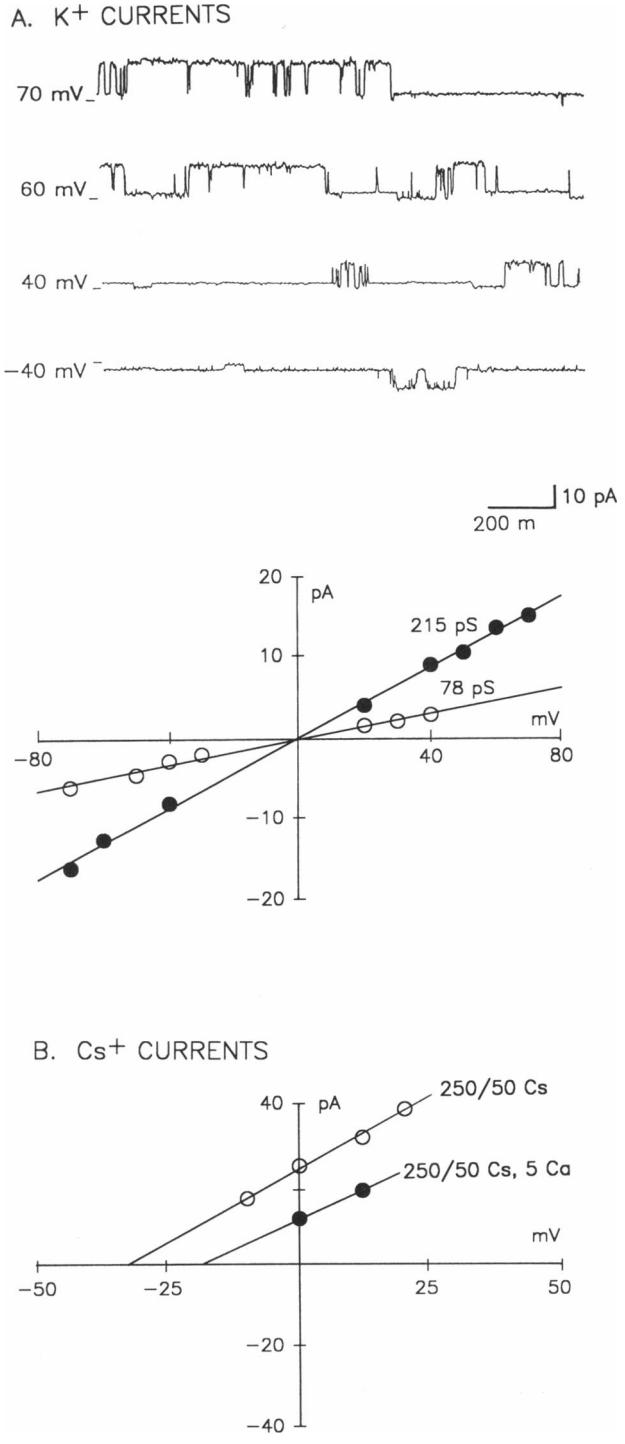

FIGURE 3 Single-channel recordings and current–voltage relations of the purified *C. elegans* ryanodine receptor. (*A*) Single-channel recordings at the indicated voltages in *cis* and *trans* solutions containing 250 mM KCl, 10 mM Hepes-Tris, pH 7.2. The open-channel conductance was contributed by two conductance states of 215 pS and 78 pS. The current–voltage relationships of the conductance states in *cis* and *trans* 250 mM KCl are shown below traces. (*B*) Current–voltage relationship of the large conductance state in *cis* 250 mM $CsSO_4$ and *trans* 50 mM $CsSO_4$ (*open circles*) and following trans addition of 5 mM $CaCl_2$ (*filled circles*). Extrapolated reversal potential before and after addition of trans $CaCl_2$ was −34 mV and −19 mV, respectively. All recordings were low-pass filtered at 300 Hz and sampled at 1 kHz.

monovalent and divalent cations was analyzed in Fig. 3 *B* in gradients of Cs^+ and Ca^{2+}. In these experiments we focused exclusively on the large conductance state, since it was the most easily identified. In a gradient composed of *cis* 250 mM Cs_2SO_4 and *trans* 50 mM Cs_2SO_4 (E_{Cs} = −41.5 mV, E_{SO4} = +20.8 mV) the slope conductance was 600 pS and the extrapolated reversal potential was −35 mV. The channel was thus selective for cations over anions, and from the Goldman–Hodgkin–Katz (GHK) equation we estimated $P_{Cs}/P_{SO4} \geq 20$. The permeability to Ca^{2+} was estimated in the same experiment after addition of 5 mM $CaCl_2$ to the *trans* chamber. The reversal potential shifted in the direction of E_{Ca} (positive direction), from the value dominated by the Cs^+ gradient, to

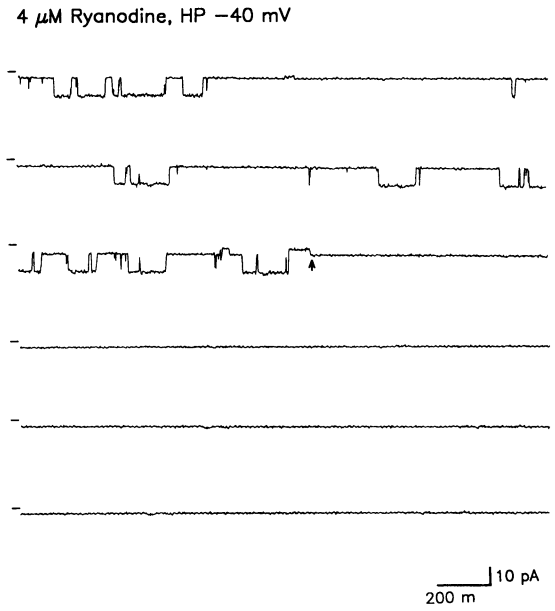

FIGURE 4 Ryanodine activation of the nematode ryanodine receptor channel. Control activity in *cis* and *trans* solutions composed of 250 mM KCl and 10 mM Hepes-Tris, pH 7.2. Ryanodine (4 μM) was added to both *cis* and *trans* chambers. Conductance change indicated by the arrow occurred 60 s after alkaloid addition.

approximately −20 mV. Assuming $P_{SO4} = P_{Cl} = 0.05$, the GHK equation yielded a $P_{Ca}/P_{Cs} = 4$, which is similar to that reported for the ryanodine receptor of rabbit skeletal muscle (10). Both the Cs^+ and the K^+ conductances were lower than those reported for the main conductance level of the mammalian skeletal, cardiac, or brain channels (7–10).

The effect of 4 μM ryanodine on the sucrose gradient-purified *C. elegans* channel is shown in Fig. 4. The alkaloid induced an abrupt change in kinetics (see *arrow*) that stabilized the 78-pS conductance state and blocked the 215-pS state. This result is consistent with reports in muscle and brain. In skeletal muscle, ryanodine induces a low-conductance state that remains permanently open (10); in cardiac muscle, the low-conductance state is closed after long exposure to alkaloid (8); whereas in brain, the alkaloid only induces a closure of the channel without generating a long-lived low-conductance state (9). The nematode channel would appear to be intermediate to cardiac and brain channels. Yet, it is not immediately clear how the effect of ryanodine on the purified nematode channel would explain paralysis of this organism in vivo. In Fig. 4, the open probability of the 78-pS state was 0.97 during the control period and increased to 1.0 in the presence of ryanodine. On the other hand, the open probability of the 215-pS state decreased upon addition of ryanodine from 0.34 to 0. When changes in both states are taken together, there is a net decrease in open probability, whereas in mammalian muscle short-term exposure to ryanodine produces a considerable increase in open probability (8, 10).

We recently obtained a partial cDNA homologous to mammalian ryanodine receptors from the *C. elegans* genome sequencing project (23). This 1.25-kb clone encodes a protein that is 30–40% identical to mammalian ryanodine receptor proteins between residues 3300 to 3700. The cDNA hybridizes to a large (>10 kb), moderately abundant mRNA on northern blots (E. Maryon, unpublished results). Molecular characterization of the *C. elegans* ryanodine receptor gene(s) should facilitate genetic studies of ryanodine receptors in nematodes, and perhaps in other invertebrates. *C. elegans* muscle structure has been described as intermediate between classical striated muscle and smooth muscle (24). Ryanodine receptors have been recently purified from vascular smooth muscle and appear to be functionally identical to their skeletal and cardiac counterparts (25). Interestingly, the reported single-channel conductance of the smooth muscle channel in 250 mM K^+ was 360 pS (25) instead of 500 pS reported for the skeletal channel (10). Thus the smooth muscle channel and the nematode channel, which has a two-state combined conductance of 290 pS in the same salt concentration, could be structurally homologous. However, the presence of larger conductance states in the nematode receptor cannot be totally discarded at this point due to the incomplete recovery of [^{3}H]ryanodine binding sites during purification.

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